The Role of Continuous Moderate Exercise on HSP70 Expression and The Transform cell number on oral squamous cell mus musculus injected by benzopyrene

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THE ROLE OF CONTINUOUS MODERATE EXERCISE ON HSP70 EXPRESSION AND THE TRANSFORM CELL NUMBER ON ORAL SQUAMOUS CELL MUS *MUSCULUS* INJECTED BY BENZOPYRENE

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ABSTRACT

Non-communicable diseases are currently responsible for most global deaths, and cancer is estimated to get first rank as a cause of death. The prevalency of cancer is 1,4% out of 1000 people. Cancer is mainly caused by two factors: genetic and environmental, one of them being is a carcinogenic agent benzopyrene that found in cigarettes. Physical exercise can decrease blood glucose and fatty acid levels within the blood but its effect on cancer prevention, especially on caspase-3 expression, is still unknown. To prove thee role of continuous moderate exercise on Hsp70 expression and the number of transform cell on oral squamous cell Mus musculus injected by benzopyrene. 18 mice were divided into three study groups : control group 1 (K1) not given any physical exercise and benzopyrene, control group 2 (K2) not given any physical exercise but induced with 0.08 mg benzopyrene, treatment group (P) were given physical exercise in moderate intensity and induced with 0.08 mg benzopyrene. Buccal mucosa tissue samples were taken and stained for immunohistochemistry and examined under a light microscope at 400x at 10 different angles. There was no difference among the three groups on Hsp70 expression (p = 0.874), but there was significant differences among the three groups on the number of transform cell (p = 0.000). The conclusion of this study was continuous moderate exercise have no effect to Hsp70 expression, but could decrease the number of transform cell on oral squamous cell Mus musculus

KEY WORDS: Continuous Moderate Exercise, Oral Squamous Cell, Benzopyrene, Hsp70, Transform Cell

INTRODUCTION

Non-communicable diseases are currently responsible for most global deaths, and cancer is estimated to get first rank as a cause of death and the only obstacle to increasing life expectancy in every country in the world in the 21st century (Bray et al., 2018). In the United States, cancer is the second leading cause of death, and in 2019 it is estimated there are 1.762.450 new cancer cases dan 606.880 cancer deaths (Siegel et al., 2019). In Indonesia, according to Riset Kesehatan Dasar (Riskesdas) in 2018, cancer prevalence reach 1.79 per 1000 population, enhance from 2013 as much 1.4 per 1000 population. This condition make Indonesia has a rank 8th, with the most cases in Southeast Asia, and ranked 23rd in Asia (Riskesdas, 2018).

Head and neck cancer includes malignant tumors that can occur in several part of the upper digestive tract, including larynx 30.37%; lips and oral cavity 29.08%; pharynx 20.03%; salivary gland 10.94%; the rest are in other locations such as the external nose, nasal cavity and sinuses, earlobe and outer ear canal (Stoyanov et al., 2017). Head and neck cancer donate more than 900,000 cases and 370,000 deaths worldwide each year (Iwatsubol et al., 2019). Head and neck cancer is the sixth most common cancer in the world with around 630,000 new patients diagnosed each year (Vigneswaran & Williams, 2014). About 76.74% are

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squamous cell carcinomas; 6.14% adenocarcinoma, the remainder is malignancy in the primary tonsillar epithelium, mucous lymphoid tissue or parenchymal lymphoma, connective tissue neoplasia, also neuroendocrine and vascular malignancies (Stoyanov et al., 2017). But according to Choi & Myers (2008), 90% head and neck cancer is oral squamous cell carcinoma (OSCC). The rate of OSCC increases among young white individuals aged 18 to 44 years, especially among white women. The 5-year survival percentage for patients with OSCC varies from 40-50% (Markopoulos, 2012).

Cancer can caused by many factors, such as a person's genetics, environment, infection and lifestyle (Irmawati[1] et al., 2018). Risk factors for oral cancer include squamous cell carcinoma, among others: cigarettes, alcohol, inflammation, infection, preneoplasia, consumption of marijuana, nutritional deficiencies, denture irritation, mouthwash, dental plaque, candidiasis, diabetes mellitus, and viruses (Tanaka et al ., 2011). Cigarettes are pollutants that are often referred to as the highest risk factors for the onset of squamous cell carcinoma (Jiang et al., 2019). The negative impact of cigarettes has often been conveyed in scientific studies and various media, but the number of smokers continues to grow over time. Currently Indonesia is included in the group of countries with the third highest cigarette consumption in the world after China and India (Hanafiah, 2018).

One of the ingredients contained in cigarettes is benzopyrene. Benzopyrene or Benzo[a]pyrene is a chemical compound of the hydrocarbon group obtained from incomplete combustion of organic matter. Benzopyrene can be directly absorbed by inhalation, through the food we consume and exposure to the skin. Negative effects of benzopyrene, including carcinogenesis, teratogenesis, neurotoxicity and immunotoxicity (Min et al., 2011).

Treatment given to cancer patients is often ineffective, because generally patients are detected suffering from cancer at an advanced stage, so it is important to think about preventive action. As has been done by Irmawati2 (2018), moderate intensity physical exercise can increase the ratio of Bax / Bcl-2, so that cancer cells can not be formed. Therefore this study aims to prove the role of continuous moderate exercise on expression of Hsp70 and the number of transform cells on oral squamous cell Mus musculus injected with benzopyrene.

MATERIALS AND METHODS

Ethical approval

This research has obtained ethical approval from the Board for Animal Experiments at the Faculty of Dental Medicine, Universitas Airlangga, No. 156/ KKEPK.FKG/VIII/2016.

Samples

The sample of this study was Swiss Webster (Mus musculus) strain (Balb/c), male sex, age around 2 months, and have body weight 25-35 g. The sampling technique in this study was carried out by simple random method. The sample size was 6 for each group. Samples were divided into three groups, namely control group 1 (K1), control group 2 (K2), and treatment group (K3).

Experimental design

This research was a laboratory experimental study with a posttest only control group design. This study was conducted in three different laboratories. Preparation of experimental animal models, maintenance and treatment of experimental animals was carried out in the Department of Biochemistry, Faculty of Medicine, Universitas Airlangga. Preparation and immuno histochemical examination were carried out in the Electron Microscope unit and the Integrated Laboratory, Faculty of Medicine University Airlangga.

Experimental animals were divided into three groups, namely: Control group 1 (K1), control group 2 (K2), treatment group (K3). In K1: mice are immersed in clean water, with 70% of the time from the maximum swimming capacity (MSC), 3 times per week for 12 weeks. At week 5, inducted by the oleum olivarum was 0.04 ml per kg BW, in the buccal mucosa of the upper right oral cavity, 3 times a week for 4 weeks. In K2: mice are immersed in clean water, with 70% of the time of MSC, 3 times per week for 12 weeks. At 5 weeks induced 0.08 mg benzopyrene (Sigma-Aldrich, Saint Louis, Missouri, USA) / 0.04 mL oleum olivarum per Kg BB in the buccal mucosa of the upper right oral cavity, 3 times a week for 4 weeks. In K3: mice were given swimming training with a load of 3% body weight (BB), with a time of 70% of the MSC, 3 times per week for 12 weeks. At week 5, benzopyrene induced / 0,04 mL of oleum olivarum per kg BW, on the right buccal mucosa of the upper right oral cavity, 3 times a week for 4 weeks (Irmawati[1] et al., 2018).

Hsp70 expression and number of transformed cell

In the first week 13, all animal samples were anesthetized with ether before being sacrificed to obtain a sample of upper right buccal mucosa tissue. The samples were then fixed in 10% buffered formalin and made into a paraffin block. Immunohistochemistry staining was performed using monoclonal Hsp70 antibodies from Dako (Agilent, Santa Clara, and USA). Hematoxylin and Eosin staining was performed using HE staining kit (Sigma-Aldrich, USA). The expression of Hsp70 and the number of transform cell were subsequently calculated under a light microscope

(Olympus, Tokyo, and Japan) at 10 different visual fields at 400× magnification.

Statistical analysis

The data obtained were analyzed by means of SPSS version 20 (IBM, New York, and USA) using an Kruskal Wallis test to determine the differences between groups on Hsp70 expression. While the difference of transform cell number determine with Brown-Forsyte test continued by multiple comparison Games-Howell test with significancy 0.05

RESULTS

Table 1. The difference test result of Hsp70 expression with Kruskal Wallis.

Groups	n	Hsp70				Kruskal-Wallis (p)
		Mean	SD	Min	Max	
K1 (Control 1)	6	0.15	0.14	0.00	0.30	0.874
K2 (Control 2)	6	0.20	0.31	0.00	0.80	
K3 (Treatment)	6	0.17	0.14	0.00	0.30	

Table 2. The difference test result of number of transform cells with Brown-Forsythe.

Groups	n	Number of transform cell				Brown Forsythe (p)
		Mean	SD	Min	Max	
K1 (Control 1)	6	3.17[a]	1.17	2	5	0.000*
K2 (Control 2)	6	26.17[c]	6.31	18	34	
K3 (Treatment)	6	8.33[b]	2.42	4	11	

Note : * significancy : α=0,05

[abc] =The same superscript showed no different between group (based on multiple comparisons Games- Howell)

Figure 1. The mean of cell that expressed Hsp70.

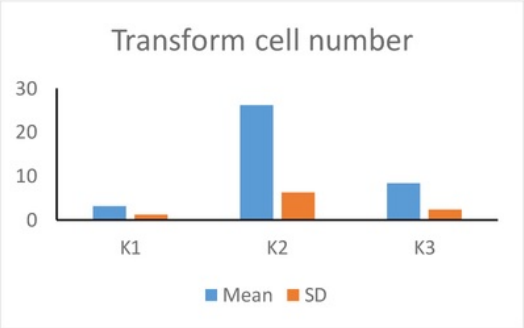

Figure 2. The mean of transform cell number.

DISCUSSION

The cancer incidence (including oral squamous cell carcinoma) has enhance every year. This is related with increasing consumption of cigarettes, now Indonesia ranks the 3rd largest cigarette consumer in the world. Cancer is a condition in which new growth occurs due to the proliferation of abnormal cells continuously, it has an ability to attack and damage other tissues.

Based on table 1, it shows p value = 0.874; which means there was no difference between the three groups, although if we observe at the mean and SD there was an increase in Hsp70 expression in K3 (0.17 ± 0.14) compared to K1 (0.15 ± 0.14). This means that swimming exercise of moderate intensity has no effect on the expression of the Hsp70 protein. This was not in accordance with Tkacova & Angelovicova's (2012) statement, that Hsp70 concentration can increase due to exercise, because exercise can activate Src and Ras-GAP signal transduction, both of which will stimulate Hsp70 mRNA transcription, which will continue by translating and forming Hsp70 proteins. The expression of Hsp70 at K3 which was no higher than K2 might be due to benzopyrene not only damaging the wild p53, but also damaging the repair gene Hsp70. Although the expression of Hsp70 on K3 is not higher than K2, its activity is still high, this can be seen in the mutant p53 expression at lower K3 and was significantly difference from K2 (Irmawati[3] et al., 2019).

Heat shock protein (Hsp) is a large class protein, found in prokaryotes and eukaryotes, and has an important role in protein homeostasis. Hsp can be found in all parts of the cell, such as the nucleus, cytosol, mitochondria, endoplasmic reticulum and others. There are several Hsp families, such as Hsp90 which are important for the formation of steroid receptor complexes, Hsp60 plays a role in protein stability, Hsp70 is needed for protein synthesis, translocation, chaperoning and folding for nascent polipeptide, and degradation of proteins undergoing aggregation (Naughton et al., 2006).

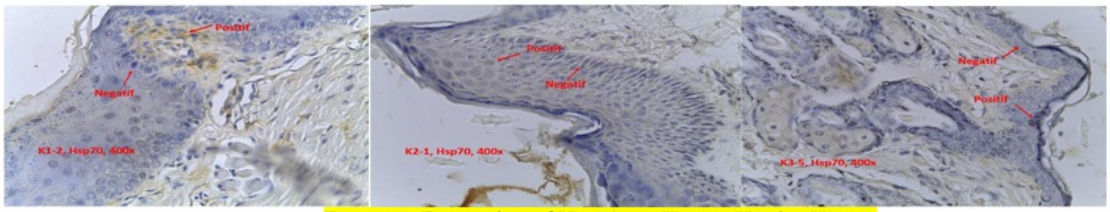

Figure 3. Expression of Hsp70 on K1 (a), K2 (b), K3 (c).

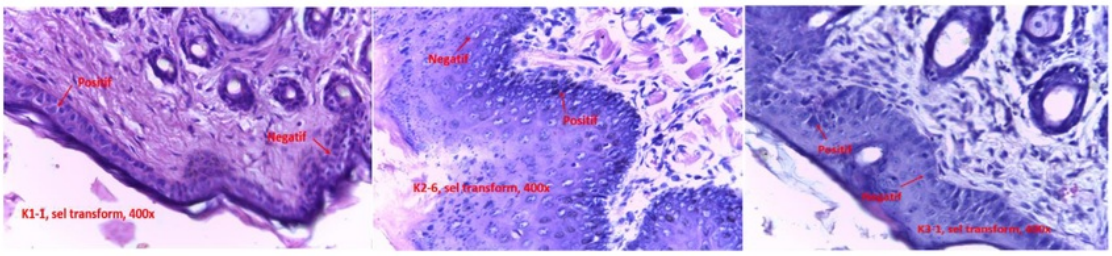

Figure 4. HPA of squamous cell epithel rongga mulut K1 (a), K2 (b), dan K3 (c).
Appears abnormal keratin formation, squamous epithelial cell proliferation, cell shape and irregular size, basophilic cell nucleus, with HE coloring.

The role of Hsp70 on repair DNA is indirectly, namely by activating the enzymes involved in the repair base excision repair (BER) path (Mendez et al., 2003). The BER path is a pathway to repair damaged nucleotide residues, known as single strands breaks (SSBs). BER is started by DNA glycosylase which releases a damaged base so that a part of DNA that no longer contains purine or pyrimidine (apurinic –apyrimidinic = AP site) is formed. Damaged bases are removed by at least one of the 10 DNA glycosylases (Chaudry MA, 2007).

The AP site whose existence is only temporary, it then truncated at 5'site (tip 5) by human apurinic-apyrimidinic endonuclease (HAP1) or also called REF1, leaving 3'OH and a single strand gap. The gap was then filled through deoxyribophosphodiesterase (dRPase) activity from DNA polymerase β, ie cutting site 3 'from the AP site, then removing the remaining glucose. Furthermore, β polymerase fills the gap through covalent attachment with new nucleotides on the 3'OH site. The last ligation is done to close the base place that has been removed (Mendez et al., 2003).

Hsp70 stimulates 10-100 times HAP1 activity, while also stimulating β polymerase activity, among others through the formation of direct Hsp70-polymerase β bonds, or through the N-terminal Hsp70 section. The ability of Hsp70 to stimulate β polymerase is higher than other proteins (Mendez et al., 2003; Kresno, 2012).

In K2, the expression of Hsp70 was higher (0.20 ± 0.31) compared to K3. This is possible because benzopyrene can trigger the expression of Hsp70, according to the statement of Naughton et al. (2006), that the expression of Hsp70 can be stimulated due to several conditions, such as hyperthermia, ischemia, changes in pH, lack of energy, and abnormal protein formation (the presence of defective / abnormal genes, so abnormal proteins are formed as well). But this is contrary to the research conducted by Marfe et al. (2010) that running a marathon can increase the expression of Hsp70.

On K1, Hsp70 expression also appeared (0.15 ± 0.14), but it was lower than K3, this could be due to the limitations of this study, where limited (not too broad) research sites allowed subjects who actively smoked without intentional. This condition allows the exposure of cigarette smoke passively (at the research site), so it still provides an opportunity for interference with DNA (DNA mutations), as a result the body is stimulated to perform DNA repair, one of which is by increasing the synthesis of Hsp70 repair genes.

The results of this study are incompatible with the research conducted by Noble et al. (2006), which states that rat experimental animals treated with runmil for 6 minutes can increase Hsp70 expression in the gastrocnemius musculus. Also research by Thompson et al. (2003) that running downhill with a slope of 10° for

30 minutes in untrained subjects can increase Hsp70 response after 48 hours post exercise.

Based on table 1 and figure 2, the results of this study indicate that moderate intensity swimming exercise can inhibit transform cell formation. This can be seen in the mean and SD number of transform cells in K3 (8.33 ± 1.17) which is much lower than K2 (26.17 ± 6.31), while in K1 the transformed cell should not be formed, apparently detected on HPA examination.

In this study the detection of transform cells in K1 can occur due to the limitations of this study, which is not possible to do sterilization at the study site. The study area is not too broad, accidentally made cigarette smoke inhaled by experimental animals in this study. Cigarette smoke containing carcinogenic ingredients benzopyrene can enter the body through various ways, namely through the airways (inhalation), the digestive tract (ingestion), and the skin. Benzopyerene is rapidly distributed to a number of tissues, such as the kidneys, small intestine, trachea, stomach, testis, esophagus and liver (PHE, 2008).

In hepatocytes, benzopyrene is first oxidized by the cytochome P450 enzyme to produce several products, including Benzo (a) pyrene 7.8-oxide; then Benzo (a) pyrene 7,8-oxide undergoes hydration by epoxide hydrolase 1 enzyme which opens epoxide ring to produce Benzo (a) pyrene 7,8-dihydrodiol. Next Benzo (a) pyrene 7,8-dihydrodiol is oxidized by the CYP450 enzyme to produce diol epoxide, namely Benzo (a) pyrene-7,8-dihydrodiol, 10-epoxide (BPDE). BPDE is the main metabolite that is toxic and carcinogenic (Rekhadevil et al, 2014).

Benzopyrene is very easy to cross cell membranes and quickly moves into cells to give mutagenic and carcinogenic effects, because flat molecules are formed from a combination of benzene-like rings and have a low solubility in water, contain lots of carbon and are hydrophobic. Benzopyrene can form covalent bonds with nucleophilic guanine nucleotide bases at N2 position. The BPDE bond with DNA forms a structure called benzopyrene-DNA adduct, this structure can affect DNA by changing DNA replication (copying DNA during cell division) (Teach, 2007). The bond of BPDE with DNA will cause mutations in DNA, which is a signal to cells to synthesize more repair genes, such as GADD45 which repairs via the NER line or Hsp70 which repairs via the BER path, with the hope that the defective gene is still can be repaired to return to normal, so as not to cause interference to the cell. However, if the GADD45 and Hsp70 repair genes are no longer able to repair because of the damage experienced by an unrepairable gene, then this condition will signal to the body to activate other pathways to eliminate the damaged gene.

Repair failure will give a signal to the genes involved in apoptosis, especially p53. p53, also called wild p53, which is called the guardian of genes, has a central role in eliminating defective genes, both by stimulating the G1 phase of the cell cycle that causes cell cycle arrest and by activating other genes involved in apoptosis. The increase in Bax gene expression due to swimming exercise of moderate intensity can be interpreted as an increase in Bax gene activity. Increased activity of the Bax gene can inhibit Bcl-2 activity, resulting in changes in the permeability of the mitochondrial membrane. This results in the release of cytochrome-c into the cytoplasm, cytochrome-c will activate Apaf-1, and with Apaf-1 it will form an apoptosome complex, which later this complex will activate caspase 9 as an apoptosis initiator, caspase 9 will activate caspase 3 as the executor apopotosis, so that apoptosis can take place. When apoptosis occurs, the mutant p53 gene will decrease in expression and the number of transform cells will also decrease.

Wild p53 can also control the cell division cycle by stimulating the p21 gene transcription process. The increased expression and activity of p21 will inhibit all CDK (CDK-4 and CDK-6 in phase G1; CDK-2 in phase S; CDK-1 in phase M). If the CDK does not function, the cyclin-CDK complex is not formed so that phosphorylation inhibits the Rb gene, which continues with the cessation of the cell cycle (Kresno, 2012).

One result that can be caused by the accumulation of benzopyrene is cancer. Cancer occurs due to genes that have mutations. Our body has regulation of any changes that occur in cells. The presence of mutant DNA is responded by the body in two ways, namely if repairable DNA damage, it is still possible to do repair by repair genes such as heat shock protein 70 (Hsp70). Hsp70 is one of the Hsp isoforms that plays a major role in physiological mechanisms. Hsp70 is involved in protein synthesis, ion transport, protection from protein denaturation and aggregation, as well as repairing damaged proteins so that they can function again. Kowalchuck (2013), in his study concluded that acute exercise in high intensity rats (treadmill 30 m / minute) can increase the expression of Hsp70 and Hsp90 higher compared to acute low intensity exercise (treadmill 15 m / minute).

If the DNA damage is not possible to repair, the body will eliminate the mutant cells through the apoptotic pathway. Apoptosis is programmed cell death, which can occur both physiologically and pathologically (Merkle, 2009; Mohan, 2010). Apoptosis is regulated by pro-apoptotic genes and anti-apoptotic genes. The main gene of apoptosis is called the guardian of the genome, namely p53 is a tumor suppressor gene (George, 2011). Apoptosis can occur when the wild gene p53 can work optimally. However, when wild p53 mutates into mutant p53, apoptosis will be disrupted.

CONCLUSION

The conclusion of this study was continuous moderate exercise have no effect to Hsp70 expression, but could decrease transform cell number on oral squamous cell Mus musculus that injected by benzopyrene.

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